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Involvement of calcineurin-dependent degradation of Yap1p in Ca^{2+}-induced G_2 cell-cycle regulation in *Saccharomyces cerevisiae*

Hiroshi Yokoyama, Masaki Mizunuma, Michiyo Okamoto, Josuke Yamamoto, Dai Hirata & Tokichi Miyakawa[+]
Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Japan

The Ca^{2+}-activated pathways in *Saccharomyces cerevisiae* induce a delay in the onset of mitosis through the activation of Swe1p, a negative regulatory kinase that inhibits the Cdc28p/Clb complex. We isolated the *YAP1* gene as a multicopy suppressor of calcium sensitivity owing to the loss of *ZDS1*, a negative regulator of *SWE1* and *CLN2* gene expression. *YAP1* deletion on a *zds1*Δ background exacerbated the Ca^{2+}-related phenotype. Yap1p was degraded in a calcineurin-dependent manner when cells were exposed to calcium. In *yap1*Δ cells, the expression level of the *RPN4* gene encoding a transcription factor for the subunits of the ubiquitin–proteasome system was diminished. The deletion of *YAP1* gene or *RPN4* gene led to the accumulation of Swe1p and Cln2p. Yap1p was a substrate of calcineurin *in vivo* and *in vitro*. The calcineurin-mediated Yap1p degradation seems to be a long adaptive response that assures a G_2 delay in response to a stress that causes the activation of the calcium signalling pathways.

Keywords: calcium; calcineurin; Swe1p; Yap1p; yeast
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INTRODUCTION

Calcium-triggered signalling mechanisms are used by virtually all eukaryotic organisms to regulate a wide variety of cellular processes, including gene expression. Transient increases in cytosolic Ca^{2+} result in the activation of diverse enzymes, including calcineurin, a highly conserved Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase.

In the yeast *Saccharomyces cerevisiae*, calcineurin has been implicated in stress-induced gene expression, ion homeostasis and viability after exposure to mating pheromone (for reviews, see Aramburu *et al*, 2000; Cyert, 2001). More recently, we demonstrated that calcineurin and the Mpk1p pathway, in a coordinated fashion, activate Swe1p, a negative regulatory kinase of the Cdc28p/Clb complex (Booher *et al*, 1993), leading the cell to a cell-cycle delay in the G_2 phase (Mizunuma *et al*, 2001). The activation of these pathways is thought to occur in response to membrane stretching (Mizunuma *et al*, 1998).

The effect of calcium on cell-cycle regulation is conspicuous on a *zds1*Δ background lacking the negative regulator for *SWE1* and *CLN2* transcription (Ma *et al*, 1996; Mizunuma *et al*, 2004). Previously, we showed that the cell cycle of *zds1*Δ strain cultivated in medium containing a high concentration of $CaCl_2$ was delayed in the G_2 phase and that this strain showed polarized bud growth owing to the activation of the cellular calcium signalling pathways (Mizunuma *et al*, 1998). To identify the proteins that bear Zds1p-like functions in the calcium-induced G_2 cell-cycle regulation, we screened for genes the overexpression of which could suppress the calcium phenotypes of the *zds1*Δ strain. By this screening, the *YAP1* gene was obtained as a suppressor. Here, we show that the Swe1p and Cln2p levels were negatively regulated indirectly by Yap1p. On exposure of cells to exogenous calcium, Yap1p was degraded in a calcineurin-dependent manner, which led to transcriptional downregulation of the *RPN4* gene encoding a transcription factor that modulates the expression levels of the genes involved in the ubiquitin–proteasome system. The Yap1p degradation, which eventually leads to Swe1p and Cln2p upregulation, was suggested as a mechanism to assure a G_2 delay in response to a stress that causes the activation of calcineurin.

RESULTS

YAP1 gene is a high-copy suppressor of *zds1*Δ strain

To investigate the detailed mechanism of the calcium-induced cell-cycle regulation, we carried out a screening for genes the overexpression of which could suppress the calcium sensitivity of the *zds1*Δ strain. The genes contained in the suppressor plasmids were classified into 19 groups, including *ZDS1* and its homologue *ZDS2* (Bi & Pringle, 1996; Yu *et al*, 1996). Excluding these two genes, a representative plasmid was chosen from each of the other groups, and they were designated as pSUZ1∼17 (suppressor of zds1). By sequencing of the inserts, plasmids containing the genes, such as *PMR1*, *PMC1* and *MIH1*, were identified, showing the

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan
[+]Corresponding author. Tel: +81 82 424 7763; Fax: +81 82 424 7763;
E-mail: tmiyaka@hiroshima-u.ac.jp

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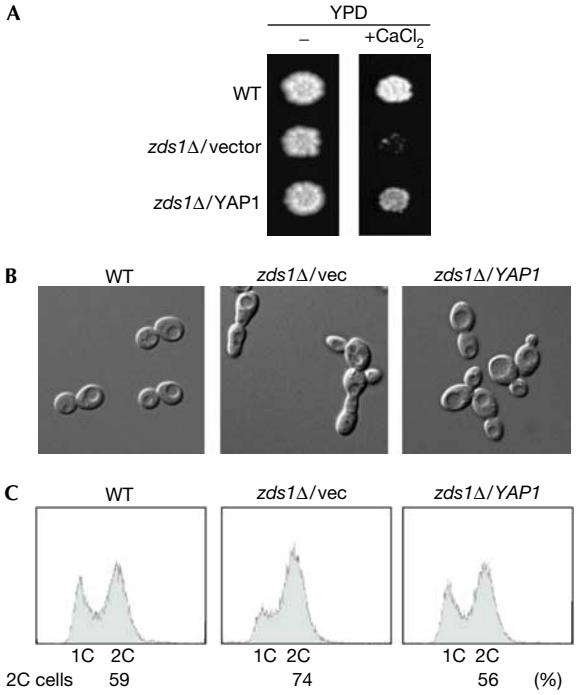

Fig 1 | Overexpression of *YAP1* gene suppresses various calcium sensitivity phenotypes of *zds1*Δ strain. (**A**) Wild-type (WT) and *zds1*Δ strains transformed with empty plasmid (pYI1) or pYI1-YAP1 plasmid were spotted on YPD plates with or without 250 mM $CaCl_2$ and incubated for 2 days at 25 °C. (**B**) Cell morphology after 6 h of incubation in YPD containing 50 mM $CaCl_2$ at 28 °C. (**C**) Fluorescence-activated cell sorting profile of propidium iodide stained cells prepared as described in (**B**) (1C, one DNA copy; 2C, two DNA copies). The percentage of the 2C cells is shown.

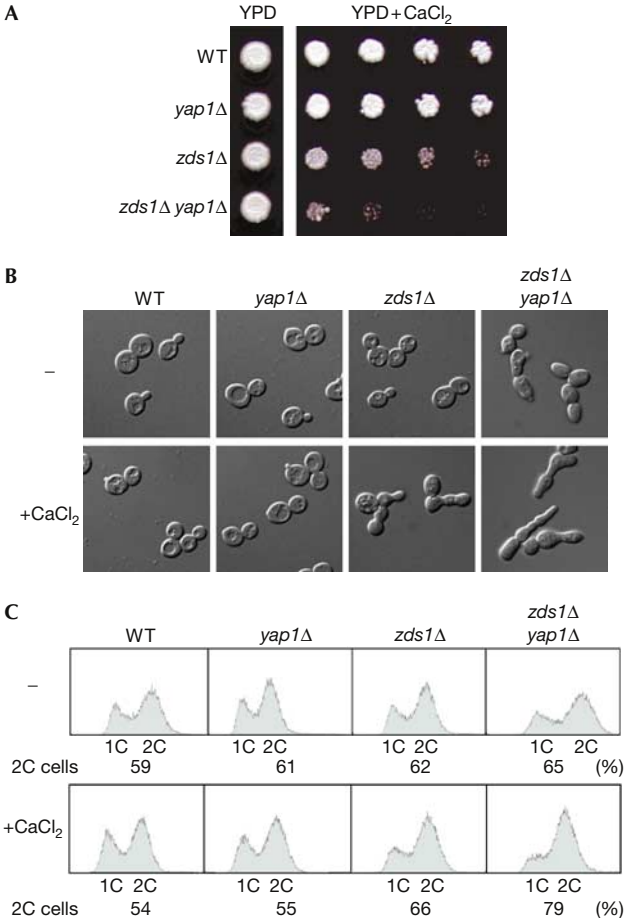

Fig 2 | Calcium sensitivity phenotypes of *zds1*Δ strain are exacerbated by deletion of *YAP1* gene. (**A**) Cells of wild-type (WT), *yap1*Δ, *zds1*Δ and *zds1*Δ *yap1*Δ strains were spotted on YPD plates either supplemented or not supplemented with 200 mM $CaCl_2$ at 25 °C (2 days). (**B**) Cell morphology after 6 h of incubation in YPD with or without 100 mM $CaCl_2$ at 28 °C. (**C**) Fluorescence-activated cell sorting profiles of propidium iodide (PI)-stained cells. The cells were prepared as described in (**B**). Percentage of the 2C cells is shown.

validity of this screening. By subcloning of the pSUZ14 plasmid, the *YAP1* gene, which encodes a mammalian AP-1-like protein (Moye-Rowley *et al*, 1989), was identified as the gene responsible for suppression. As shown in Fig 1, the growth of the *zds1*Δ strain in the $CaCl_2$-containing medium was severely inhibited, exhibiting a G_2 delay and highly polarized bud growth. Overexpression of the *YAP1* gene partially suppressed all of these calcium-induced phenotypes. So we further investigated the mechanism by which Yap1p modulated the calcium-dependent cell-cycle regulation.

Deletion of *YAP1* gene exacerbates Ca^{2+} sensitivity

To verify the contribution of Yap1p in suppressing the various calcium-induced phenotypes of the *zds1*Δ strain, we examined the effect of a *YAP1* deletion mutation on these phenotypes (Fig 2A–C). The *yap1*Δ mutation by itself did not lead to significant increases in the calcium sensitivity. However, the growth defect of the *zds1*Δ strain on calcium plates was exacerbated by further *yap1*Δ mutations (Fig 2A). Morphologically, the *yap1*Δ *zds1*Δ double deletion cells, in comparison with the *zds1*Δ cells, showed a slightly more elongated shape in YPD medium (without added $CaCl_2$), and showed a more pronounced elongated morphology than the *zds1*Δ cells in the presence of $CaCl_2$ (Fig 2B). Fluorescence-activated cell sorting analysis of the cellular DNA content demonstrated that the *yap1*Δ *zds1*Δ strain, in comparison with the respective single deletion strains, suffered from a severe defect in G_2 progression in the presence of $CaCl_2$ (Fig 2C). These results indicated that Yap1p and Zds1p may share a common biological function in parallel pathways in calcium signalling.

Yap1p degradation is calcineurin dependent

The results of the genetic experiments indicate that Yap1p is involved in the regulation of the calcium-induced G_2 delay and polarized bud growth. We examined whether the levels of *YAP1* messenger RNA and Yap1p were altered by calcium. For this purpose, we used the strain carrying a chromosomally integrated construct for Myc-epitope-tagged Yap1p at the carboxyl terminus under the control of its own promoter. The Myc-tagged Yap1p suppressed the elongated bud growth of the *zds1*Δ *yap1*Δ strain in the presence of 100 mM $CaCl_2$, suggesting that the construct is functional *in vivo* (data not shown). We previously noted that the

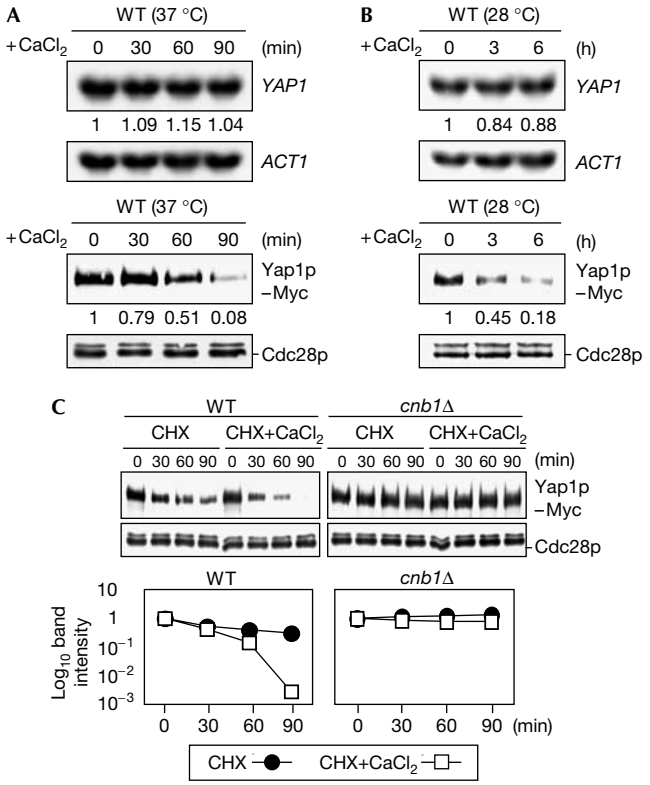

Fig 3 | Yap1p level is downregulated by calcium post-translationally in a calcineurin-dependent manner. (**A**) *YAP1* and *ACT1* messenger RNA levels in wild-type (WT) strain were determined by northern blotting (upper). The bands of Yap1p–Myc and Cdc28p were detected by western blotting (lower). Early log-phase growing cells were suspended in YPD containing 100 mM $CaCl_2$ and incubated at 37 °C. The intensity of *YAP1* or *ACT1* mRNA was measured using a BAS-1800 bio-imaging analyser (FUJI FILM) and the *YAP1* level was normalized to the *ACT1* mRNA level. Yap1 protein level was quantified with an Image SXM (Surface Science Research Center, University of Liverpool, UK) and normalized relative to the Cdc28p level of each lane. (**B**) Same as (**A**), except that the incubation was carried out at 28 °C. (**C**) Early log-phase growing cells of WT and *cnb1*Δ strains were suspended in YPD plus 100 μg/ml cycloheximide (CHX) either supplemented or not supplemented with 100 mM $CaCl_2$ and incubated at 37 °C. Yap1p–Myc and Cdc28p were detected by western blotting. The amount of Yap1p was quantified as described for (**A**).

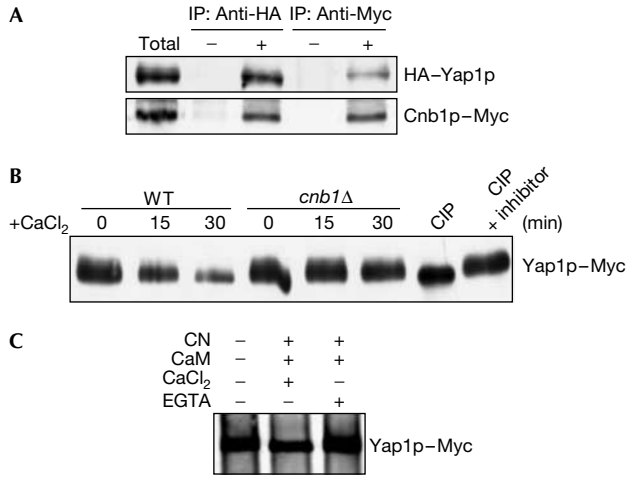

Fig 4 | Phospho-Yap1p is a substrate of calcineurin. (**A**) *cnb1*Δ *yap1*Δ strain co-transformed with CTF-CNB1–Myc and YCp50-YAP1–HA (haemagglutinin) was grown in YPD medium at 28 °C until early log phase. Cell extracts were processed for immunoprecipitation with anti-HA antibody or anti-Myc antibody. Total and immunoprecipitated proteins were detected by western blot analysis. (**B**) Early log-phase growing cells of wild-type (WT) or *cnb1*Δ strain were suspended in YPD with or without 100 mM $CaCl_2$ at 37 °C, and samples were taken at the indicated time periods. Cell extracts of *cnb1*Δ cells were treated with λ-phosphatase (calf intestinal alkaline phosphatase) for 30 min with or without sodium orthovanadate (inhibitor). Yap1p–Myc was detected by western blotting. (**C**) Extracts were prepared from *cnb1*Δ cells. Immunoprecipitated Yap1p–Myc was treated with recombinant calcineurin (CN), calmodulin (CaM), $CaCl_2$ and EGTA as described in the supplementary information online. The samples were subjected to SDS–polyacrylamide gel electrophoresis, and Yap1p–Myc was then detected by western blotting.

calcium-induced, proteasome-dependent degradation of Hsl1p proceeded more rapidly at 37 °C than at 28 °C (Mizunuma *et al*, 2001). Thus, we determined the changes in the levels of *YAP1* mRNA and Yap1p at 37 °C following the shift of wild-type cells to the medium containing $CaCl_2$. Although the *YAP1* mRNA level was not altered, the Yap1p–Myc level of the wild-type strain was remarkably diminished by the presence of calcium (Fig 3A). At 28 °C, the calcium-induced decrease in the Yap1p–Myc level was observed at a slower rate (Fig 3B). To distinguish whether Yap1p was downregulated at the translational or post-translational level, we examined the effect of calcium on the stability of Yap1p–Myc in the presence of cycloheximide. The rate of the decrease in the intensity of Yap1p–Myc in the presence of the protein synthesis inhibitor was still accelerated by calcium (Fig 3C,D). Under similar conditions, the Yap1p–Myc level in cells lacking calcineurin (*cnb1*Δ) was not altered by calcium. These results indicate that the degradation of Yap1p–Myc was induced by calcium in a calcineurin-dependent manner (Fig 3C,D).

Phospho-Yap1p is dephosphorylated by calcineurin

The calcineurin dependence of Yap1p downregulation raises the possibility that Yap1p might be a substrate for calcineurin. We first examined, by immunoprecipitation experiments, whether calcineurin and Yap1p physically interacted *in vivo*. As shown in Fig 4A, Myc-tagged Cnb1p and haemagglutinin (HA)-tagged Yap1p were co-precipitated in a reciprocal manner. Co-precipitation was observed by incubation in the presence of EDTA, suggesting that the association of Yap1p and calcineurin is not induced by calcium (data not shown).

As Yap1p phosphorylation has not been well characterized previously, we examined whether Yap1p can be phosphorylated *in vivo*. When the Yap1p–Myc immunoprecipitate was treated with calf intestinal alkaline phosphatase (CIP), a band that migrated faster than that similarly treated in the presence of a phosphatase inhibitor was observed, showing that Yap1p had been phosphorylated (Fig 4B). We further examined the possibility

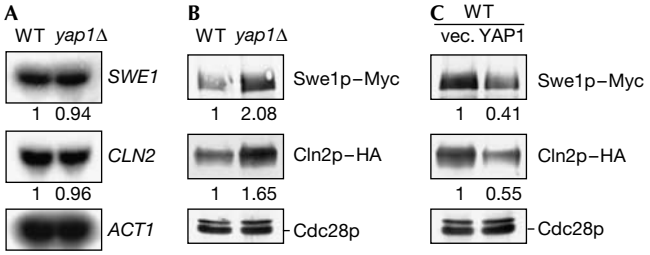

Fig 5 | Swe1p and Cln2p levels, but not their messenger RNA levels, are elevated in *yap1*Δ strain, whereas they are decreased in *YAP1*-overexpressing strain. (**A**) *SWE1*, *CLN2* and *ACT1* mRNA levels in wild-type (WT) and *yap1*Δ strains were determined by northern blotting. Samples were prepared from early log-phase growing cells at 28 °C. The amount of mRNA was quantified and normalized as described for Fig 3A. (**B**) Swe1p–Myc, Cln2p–HA (haemagglutinin) and Cdc28p were detected by western blotting using cell cultures similar to those indicated in (**A**). (**C**) Early log-phase growing cells (at 28 °C) of WT transformed with empty plasmid (vec.) or pYI1-YAP1 plasmid (YAP1) were suspended in YPD containing 100 mM $CaCl_2$, incubated for 3 h at 28 °C and then used for western blotting. The amount of protein shown in (**B**,**C**) was quantified and normalized as described for Fig 3A.

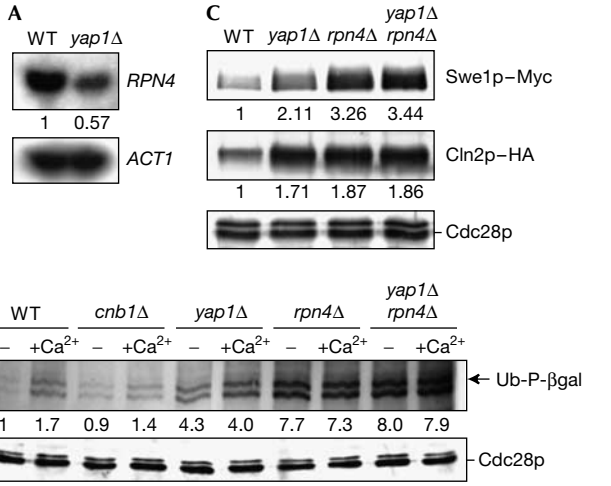

Fig 6 | Yap1p is required for the activation of *RPN4* gene expression and the latter is required for proteasome activity and elimination of Swe1p. (**A**) *RPN4* and *ACT1* messenger RNA levels in wild-type (WT) and *yap1*Δ cells were determined by northern blotting. Samples were prepared from early log-phase growing cells at 28 °C. The amount of mRNA was quantified as described for Fig 3A and normalized relative to the *ACT1* mRNA level. (**B**) Early log-phase growing cells of the indicated strains transformed with plasmid carrying a gene encoding Ub-Pro-β-galactosidase (Ub-P-βgal) were suspended in YPG with or without 100 mM $CaCl_2$ and incubated at 37 °C for 3 h. The Ub-P-βgal (indicated by an arrow) and Cdc28p were detected by western blotting. The values are means from two independent experiments. (**C**) Swe1p–Myc, Cln2p–HA (haemagglutinin) and Cdc28p in WT and *rpn4*Δ cells were detected by western blotting. Samples were prepared from early log-phase growing cells at 28 °C. The amount of protein shown in (**B**,**C**) was quantified and normalized as described for Fig 3A.

that Yap1p is a substrate of calcineurin *in vivo*. When cell extracts prepared from wild-type cells grown in calcium medium were analysed by electrophoresis, the intensity of Yap1p–Myc bands was diminished with progression of incubation time, reflecting its calcineurin-dependent degradation. The change in Yap1p intensity was accompanied by the preferential loss of the slower migrating component from the broad band seen at time 0. By contrast, no significant changes in the intensity and mobility of Yap1p–Myc were observed in the *cnb1*Δ cells. These results indicate that the faster migrating component was generated in a calcineurin-dependent manner. The CIP-treated Yap1p–Myc migrated faster than Yap1p–Myc generated in wild-type cells grown in calcium medium, suggesting that Yap1p contained other phosphorylated residues that were not removable by calcineurin. Moreover, we found that Yap1p was a direct substrate of calcineurin *in vitro* (Fig 4C). These results show that Yap1p was a direct substrate of calcineurin.

Yap1p degradation leads to a decrease of *RPN4* mRNA

Because the effects of the overexpression and deletion of the *YAP1* gene in calcium signalling resembled those of the *ZDS1* gene, we determined whether the *YAP1* gene was involved in the regulation of *SWE1* and *CLN2* transcription by northern blot analysis. However, the effect of *YAP1* deletion on the expression levels of these genes was negligible (Fig 5A).

To examine whether Yap1p functioned at the post-transcriptional level, we compared the levels of Swe1p and Cln2p in wild type and in *yap1*Δ strains carrying chromosomally integrated constructs for Myc-tagged Swe1p and HA-tagged Cln2p (Fig 5B). The levels of these proteins in the *yap1*Δ strain were significantly elevated compared with those in the wild-type strain. Contrary to the effect of *YAP1* deletion, overexpression of the *YAP1* gene led to decreased levels of these proteins (Fig 5C).

The Yap1p degradation led to the upregulation of Swe1p and Cln2p. What might be the mechanism underlying this upregulation? Recently, Yap1p, together with the Pdr1p and Pdr3p transcription factors, was implicated in the transcriptional activation of the *RPN4* gene encoding a transcription factor that modulates the expression levels of the genes for the ubiquitin–proteasome system during the oxidative stress response (Owsianik *et al*, 2002). We reasoned that the Yap1p degradation may cause downregulation of Rpn4p expression and that the decreased activity of the proteasome system may lead to Swe1p upregulation, which is normally degraded in the G_2 phase by the ubiquitin–proteasome system (Kaiser *et al*, 1998). As expected, the *RPN4* mRNA level in the *yap1*Δ cells, in comparison with that in wild-type cells, was diminished (Fig 6A).

To investigate whether the downregulation of the *RPN4* mRNA level indeed leads to the decreased activity of the proteasome, we compared the activities of the ubiquitin–proteasome of wild-type and *yap1*Δ cells by measuring the levels of the ubiquitin–β-galactosidase conjugate protein, a well-characterized proteasome substrate (Johnson *et al*, 1995). Of the doublet bands observed, the slower migrating band was shown to correspond to Ub-P-βgal (Saeki *et al*, 2004). As shown in Fig 6B, wild-type cells treated with exogenous calcium for 3 h showed a diminished activity of degrading the substrate protein, and its degradation was Yap1p dependent. No significant additive effect of *yap1*Δ mutation on the rate of protein degradation by the *rpn4*Δ strain was observed,

suggesting a Yap1p–Rpn4p linear regulatory pathway (Fig 6B,C). The degradation of the substrate protein in the *cnb1*Δ strain was still weakly but reproducibly stimulated by calcium, suggesting that other calcium-dependent mechanisms may be involved in the downregulation of the proteasome (Fig 6B). Consistent with the idea that the degradation of Swe1p and Cln2p is dependent on the proteasome-mediated proteolysis, the Swe1p–Myc and Cln2p–HA levels of the *rpn4*Δ strains were higher than those of the wild-type strain (Fig 6C). These results indicate that the downregulation of *RPN4* expression caused by the calcium-dependent Yap1p degradation may have contributed to the promotion of the G_2 delay and polarized bud growth through the upregulation of Swe1p and Cln2p levels.

DISCUSSION

Here, we discovered a novel function of Yap1p in the calcineurin-induced G_2 cell-cycle regulation (Fig 7). Yap1p has been well characterized as a central regulator of responses to oxidative stress (Balzi & Goffeau, 1994; Hirata *et al*, 1994; Kuge & Jones, 1994; Wemmie *et al*, 1994; Wu & Moye-Rowley, 1994; Kuge *et al*, 1997). We also showed a new Yap1p function in calcium signalling. The calcium sensitivity phenotypes of the *zds1*Δ strain were partially suppressed by *YAP1* overexpression, and, conversely, exacerbated by *yap1* deletion. Our genetic data indicated Yap1p to be a negative regulator of the calcium signalling that is linked to the Swe1p- and Cln2p-mediated regulation of cell cycle and morphology (Figs 1,2). The Yap1p degradation caused the accumulation of Swe1p and Cln2p through the downregulation of the ubiquitin–proteasome system, in a manner mediated by the inhibition of the *RPN4* expression. Supporting this possibility, the activity of the proteasome system, as determined by the level of the ubiqutin-conjugated β-galactosidase, was decreased by exogenous calcium in a manner dependent on the Yap1p degradation.

Previously, it was demonstrated that calcineurin interacts with Skn7p, a protein that also interacts with Yap1p, by virtue of an intermediate protein Crz1p in calcium signalling (Williams & Cyert, 2001). However, the Yap1p–Myc degradation was induced by calcium on the *skn7*Δ and *crz1*Δ backgrounds, similar to the wild-type background, suggesting that Skn7p and Crz1p do not participate in this mechanism (H.Y. *et al*, unpublished results).

We showed that Yap1p was phosphorylated by an as yet unidentified kinase(s) and dephosphorylated by calcineurin, thus identifying Yap1p as a substrate of calcineurin (Fig 4). The Yap1p degradation was required for downregulating the expression levels of the Yap1p-dependent genes, including the *RPN4* gene. In the 5′ non-coding region of the *RPN4* gene, a Yap1p response element (YRE; TTACTAA) is located at position −373 relative to the ATG translation initiation codon (Wu & Moye-Rowley, 1994). It was previously shown that the effect of the *YAP1* deletion on the activation of the ubiquitin–proteasome system was seen only in the absence of the Pdr1p transcription factor (Owsianik *et al*, 2002). Nevertheless, a decrease in the levels of *RPN4* mRNA and proteasome activity was seen on the *yap1*Δ *PDR1* background (Fig 6A,B). These different observations may be due to the difference in the strain background. Alternatively, it may be possible that Pdr1p is also degraded similarly as Yap1p.

In the Ca^{2+}-induced G_2 cell-cycle regulation, calcineurin upregulates Swe1p through two distinct but cooperative pathways: one involving the activation of *SWE1* transcription (Mizunuma *et al*, 1998) and the other the destabilization of Hsl1p, a negative regulator of the Swe1p kinase (Mizunuma *et al*, 2001). In the present study, we showed another role of calcineurin in Swe1p upregulation, that is, promotion of Swe1p accumulation through the calcineurin-mediated degradation of the Yap1p transcription factor. Thus, the Swe1p upregulation by calcium signalling seems to be regulated multilaterally to ensure the Swe1p activation by at least three distinct calcineurin-mediated mechanisms that operate on Swe1p at different levels: that is, transcriptional, post-translational and degradation. The present mechanism seems to be part of a long adaptive response as it requires degradation of the Yap1p transcription factor that leads to regulation of another transcription factor, which per se regulated the machinery involved in the degradation of Swe1p and Cln2p. The calcineurin-mediated Yap1p degradation seems to be a mechanism that assures a G_2 delay in response to a stress that causes the activation of the calcium signalling pathways.

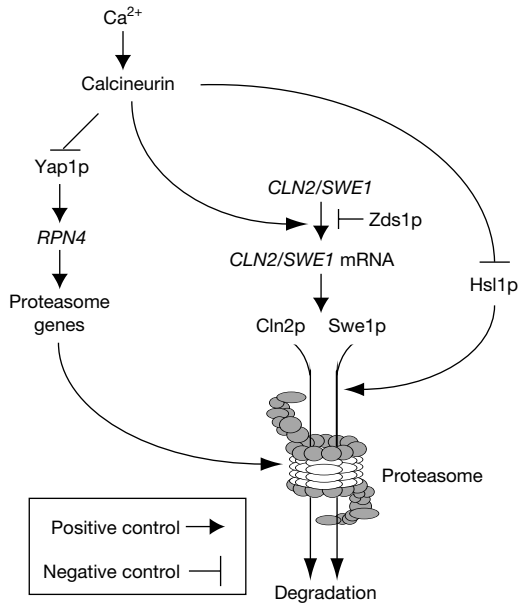

Fig 7 | Model for a novel function of Yap1p in calcineurin-dependent G_2 cell-cycle regulation. See text for details. Calcineurin is involved in sustaining the *CLN2* mRNA level as well (our unpublished data).

METHODS

Isolation of multicopy suppressors of *zds1*Δ strain. For screening, the *zds1*Δ strain was transformed with a yeast genomic DNA library constructed in the high-copy number plasmid YEp24, and the transformed cells were spread on SD minus uracil plates (8.4×10^4 cells per plate). The transformants were picked up and inoculated onto YPD plates supplemented with 300 mM $CaCl_2$, a concentration that virtually inhibited the growth of the *zds1*Δ strain. From each of 145 calcium-resistant transformants, plasmids were recovered after transformation into *Escherichia coli*. Of these, 122 plasmids reproducibly suppressed the calcium-sensitive phenotype when they were re-introduced into the *zds1*Δ strain.

General methods are available in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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